DECLARATION

I, Natsuo TANAKA of HIRAKI & ASSOCIATES, do solemnly and sincerely declare as follows:

1. That I am well acquainted with the English and Japanese languages and am competent to translate from Japanese into English.

2. That I have executed, with the best of my ability, a true and correct translation into English of Japanese Patent Application No. 150213/2001 filed on May 18, 2001, a copy of which I attach herewith.

This 23rd day of August, 2007

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SPECIFICATION

Anti-TRAIL-R antibody

FIELD OF THE INVENTION

The present invention relates to an anti-TRAIL receptor (TRAIL-R) antibody recognizing a TRAIL receptor 1 (TRAIL-R1) or a TRAIL receptor 2 (TRAIL-R2), which are cell membrane molecules involved in apoptosis.

Furthermore, the present invention relates to a prophylactic or therapeutic agent, which contains anti-TRAIL-R antibody as an active ingredient and is used against diseases caused by cells expressing TRAIL-R, and in particular relates to a therapeutic agent used against malignant tumors.

BACKGROUND OF THE INVENTION

In the living body, physiological cell death caused by normal cell alternation is referred to as apoptosis, and is distinguished from necrosis, which is pathological cell death [see Kerr, et al. (1972) Br. J. Cancer 26, 239]. Apoptosis is the phenomenon generally observed in the process of, for example, embryogenesis and the selection of lymphocytes (T cells and B cells) [see Itoh, S., et al. (1991) Cell 66, 233-243]. It is thought that when cells which should originally be eliminated by apoptosis are not removed, this may cause cancer, lupus, herpes virus infection, and other problems. Moreover, when cells that originally should survive are eliminated by apoptosis, this can cause diseases and pathological conditions such as AIDS, Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis, multiple sclerosis, retinitis pigmentosa, aplastic anemia, myocardial infarction, cerebral apoplexy or toxic substances-induced hepatopathy [see Kataoka, S., et al. (1996) The Oncologist 1, 399-401].

During apoptosis, characteristic phenomena such as curved cell surfaces, condensation of nuclear chromatin, fragmentation of chromosomal DNA, and

loss of mitochondrial function are observed. Various intrinsic and extrinsic signals are thought to cause these cellular changes. As intrinsic signals, it has been reported that oncogenes such as myc and tumor suppressor genes such as bcl-2, p53 are involved in apoptosis induction [see KATAOKA et al., (1993) JIKKEN IGAKU 11, 17, 2324-2328]. As extrinsic signals, it is known that chemotherapy drugs, radiation or the like induces apoptosis [see KATAOKA et al., (1994) SAISHIN IGAKU 49, 6, 1152-1157].

As molecules involved in such apoptosis, molecules belonging to tumor necrosis factor family (TNF family) such as tumor necrosis factor-α (TNF-α), tumor necrosis factor-β (TNF-β) and Fas ligand have been identified. TNF-α and TNF-β have been reported to induce apoptosis in carcinoma cells [see Schmid et al., (1986) Proc. Natl. Acad. Sci. 83, 1881; see Dealtry et al., (1987) Eur. J. Immunol. 17, 689]. Since mice having mutant Fas or Fas ligands develop the conditions of autoimmune disease, it has been strongly suggested that the Fas ligands have a function of eliminating self-reactive lymphocytes by apoptosis in the periphery [see Krammer, et al., (1994) Curr. Op. Immunol. 6, 279-289; see Nagata, et al., (1995) Science 267, 1449-1456]. It has been reported that agonistic mouse monoclonal antibodies that bind specifically to Fas exert apoptosis-inducing activity against carcinoma cells to the same level as that exerted by TNF-α [Yonehara, et al., (1989) J. Exp. Med. 169, 1747-1756].

These TNF family molecules transmit signals into cells by binding to specific receptors on the cell surfaces. Plural receptors for TNF family molecules are known, and they are referred to as TNF receptor family molecules.

TNF receptor family molecules are defined by the presence of cysteine-rich repetition of an extracellular domain. Among them, Fas and TNFR1, which are receptors of a Fas ligand and a TNF-α, contain within the cells a region referred to as a "death domain" sharing homology with reaper, a

Drosophila suicide gene [see Golstein, P., et al. (1995) Cell 81, 185-186; see White, K., et al. (1994) Science 264, 677-683] and such death domain is essential for signal transduction for apoptosis. Activation of Fas promotes the association of an adapter molecule FADD/MORT1 containing the death domain, and induces the activation of caspase-8 bound to FADD/MORT1. The activated caspase-8 activates downstream caspase molecules in sequence, thereby finally leading the cells to apoptosis [see Nagata, S., (1997) Cell 88, 355-365].

Recently, a novel TNF family molecule that induces apoptosis has been found. Wiley et al., [see Immunity (1995) 3, 673-682] named the molecule "TNF-related apoptosis-inducing ligand" or briefly "TRAIL." This molecule is also referred to as "Apo-2 ligand" or "Apo-2L" [see Pitt, R. M., et al. (1996) J. Biol. Chem. 271, 12687-12690]. For convenience, this molecule is referred to as TRAIL in this specification.

Unlike the Fas ligand, TRAIL is detected at a significant level in many human tissues (e.g., spleen, lungs, prostate, thymus, ovary, small intestine, large intestine, peripheral blood lymphocyte, placenta and kidney). TRAIL is constitutively transcribed in some cell lines. TRAIL has also been shown to rapidly activate apoptosis at a significantly faster pace than that induced by TNF, within a time frame resembling death signal transduction by Fas [see Marsters, S. A., et al., (1996) Curr. Biol. 6, 750-752].

Now 5 proteins have already been identified as TRAIL receptors. Two receptors, TRAIL-R1/DR4 and TRAIL-R2/DR5, have both been reported to have death domains within the intracellular regions. The transcript of TRAIL-R1 is recognized in many human tissues including the spleen, peripheral blood leukocytes, small intestine and the thymus. The transcript of TRAIL-R2 has been detected in many tissues including the spleen, peripheral blood lymphocytes and the ovary [see Pan, G., et al. (1997) Science 276, 111-113; see Pan, G., et al. (1997) Science 277, 815-818

Recombinant human TRAIL is a recombinant protein comprising the extracellular region of TRAIL, and has been reported to induce apoptosis in many types of carcinoma cells [see Griffith, T. S., et al. (1998) Curr. Opin. Immunol., 10, 559-563].

Furthermore, the recombinant human TRAIL has exerted an effect on a tumor-bearing mouse model using human colon carcinoma cells and breast carcinoma cells [see Walczak, H., et al. (1999) Nature Medicine 5, 2, 157-163]. Unlike TNF-α or FAS ligands also belonging to the TNF receptor family and having apoptosis-inducing activity, TRAIL did not provide damage to the normal tissues of mice or cynomolgus monkeys [see Ashkenazi, A., et al. (1999) J. Clin. Invest. 104, 155-162].

Based on these reports, it is thought that TRAIL selectively induces death in tumor cells. However, such selectivity has not yet been supported theoretically since TRAIL receptors are also expressed in normal cells. Moreover, the recombinant human TRAIL has recently been reported to induce apoptosis in normal human hepatocytes [see Jo, M., et al. (2000) Nature Medicine 6, No.5, 564-567] and reported to induce apoptosis also in human brain cells [see Nitsch, R., et al. (2000) The Lancet 356, 827-828]. Because agonistic anti-Fas antibodies, which induce apoptosis in hepatocytes, induce fulminant hepatitis in a very short time and thus cause death in mice and chimpanzees, cell death induction by TRAIL on hepatocytes has attracted attention as a particularly significant issue. The safety of using TRAIL as a pharmaceutical product for humans has been questioned [see Nagata, S., (2000) Nature Medicine 6, 5, 502-503].

It has also been reported that the presence or absence of the cell-death-inducing activity of TRAIL on hepatocytes depends on the type of recombinant TRAIL protein [see Lawrence, D., et al. (2001) Nature Medicine 7, 4, 383-385]. However, the safety of the recombinant TRAIL protein is still being studied.

Recently, anti-Fas antibodies that do not induce hepatopathy when administered to mice have been reported for the first time [see Ichikawa, K., et al. (2000) International Immunology 12, No.4, 555-562]. There have been no known recombinant Fas ligands confirmed not to induce hepatopathy. This suggests that antibodies having activity that may be unavailable from ligands can be obtained. However, the theoretical background of the reason that the antibodies show no hepatotoxicity in spite of inducing apoptosis in T cells has not been revealed. For example, in the case of a different antigen such as TRAIL, it has not been demonstrated whether or not agonistic antibodies having no toxicity can be obtained.

TRAIL binds to TRAIL-R1, TRAIL-R2, or both, and induces apoptosis as described above. However, via which receptor the signals to induce apoptosis in hepatocytes are introduced by TRAIL has not been shown. Furthermore, no research has been done based on the idea of whether hepatotoxicity can be avoided by adding TRAIL-R1/R2 selectivity to agonistic antibodies.

An effective therapeutic means against malignant tumors involves removing carcinoma cells and protecting normal tissues or cells. A drug whose action mechanism is apoptosis induction by the recombinant human TRAIL may cause damages to normal tissues, particularly the liver and the brain, even if it is able to remove carcinoma cells.

Currently, monoclonal antibodies such as a chimeric antibody targeting CD20, which is a receptor present on the cell membrane, and a humanized antibody targeting Her2/neu are used against malignant tumors as target diseases, and their therapeutic effects have been recognized. Since antibodies have characteristics including a long half-life in blood and high specificity to antigens, they are particularly useful as anti-tumor agents. For example, in the case of antibodies targeting tumor-specific antigens, the administered antibodies are assumed to accumulate in tumors. Thus, attack against carcinoma cells by

the immune system can be expected by complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity. In addition, the binding of a drug such as a radionuclide, a cytotoxic substance or the like to the antibodies enables the efficient delivery of the drug bound to the antibody to tumor sites. At the same time, reduced side effects can be expected due to decreased amounts of the drug having reached other non-specific tissues. When tumor-specific antigens have activity to induce cell death, antibodies having agonistic activity are administered, and when tumor-specific antigens are involved in cell proliferation and survival, antibodies having neutralization activity are administered. And then, the accumulation of tumor-specific antibodies and suppression of tumor growth or regression of tumors due to the activity of the antibodies can be expected.

It is thought to be appropriate to apply antibodies as anti-tumor agents because of the characteristics described above. In addition, if antibodies are those against TRAIL receptors, antibodies that may be obtained can avoid causing damage to the liver, which is unable to avoid with the recombinant human TRAIL, and have equivalent apoptosis-inducing activity against carcinoma cells. However, such antibodies have not been reported so far.

SUMMARY OF THE INVENTION

A first purpose of the present invention is to provide a novel antibody which has not been reported so far or a molecule analogous thereto, which is capable of binding to human TRAIL-R1 and/or human TRAIL-R2 and induces apoptosis specifically in carcinoma cells, without inducing damage to normal human hepatocytes to which a recombinant human TRAIL protein can cause damages. A second purpose of the present invention is to provide a prophylactic or therapeutic agent comprising the above antibody or a molecule analogous thereto as an active ingredient against various malignant tumors including solid tumors that are currently difficult to treat.

As a result of intensive studies on the production of antibodies against human TRAIL-R1 and -R2, we have succeeded in obtaining monoclonal antibodies from the culture supernatant by immunizing transgenic mice capable of producing human antibodies by genetic engineering techniques with human TRAIL-R1 or R2, generating hybridomas producing novel monoclonal antibodies that bind to TRAIL-R1 and/or TRAIL-R2 using the method of Kohler and Milstein et al. [see (1975) Nature 256, 495], which is generally used in monoclonal antibody production.

Furthermore, we have completed the present invention by finding that the novel monoclonal antibodies induce apoptosis specifically in carcinoma cells by binding to TRAIL-R1 and/or R2 present on the surfaces of carcinoma cells.

The present invention is as follows.

(1) An antibody or a functional fragment thereof, binding to TRAIL-R1 and/or TRAIL-R2.

The above antibody or the functional fragment thereof has at least one property selected from the following (a) to (c) of:

(a) having activity to induce apoptosis in carcinoma cells expressing TRAIL-R1 and/or TRAIL-R2;

(b) not having effect on normal human cells (except for hepatic parenchymal cells) expressing TRAIL-R1 and/or TRAIL-R2; and

(c) not inducing human hepatocyte toxicity.

In the present invention, an antibody or a functional fragment thereof having all the above properties (a) to (c) is preferred. Furthermore, the antibody or the functional fragment thereof of the present invention also includes an antibody or a functional fragment thereof that has at least one property of the above (a) to (c), and binds to TRAIL-R2, but does not bind to TRAIL-R1.

The above antibody is a monoclonal antibody produced by a mouse-mouse hybridoma, such as E-11-13, H-48-2, L-30-10, N-18-12, W-40-5, X-14-4, X-51-12 or G-3-10, and is preferably a human antibody. The type of these monoclonal antibodies are immunoglobulin G (IgG). Hybridoma H-48-2 is deposited with the accession No. of FERM BP-7599.

(2) A hybridoma producing monoclonal antibodies that bind to TRAIL-R2, which is selected from the group consisting of E-11-13, H-48-2, L-30-10, N-18-12, W-40-5, X-14-4, X-51-12 and G-3-10.

(3) A method for producing anti-TRAIL-R2 monoclonal antibodies, comprising culturing the above hybridoma and collecting the antibodies binding to TRAIL-R2 from the obtained culture product.

(4) A method for producing anti-TRAIL-R2 antibody which does not have hepatocyte toxicity, comprising selecting an antibody which does not bind to TRAIL-R1 from the population of antibodies which bind to TRAIL-R2.

(5) A prophylactic or therapeutic agent against tumors, comprising as an active ingredient the above antibody or the functional fragment thereof.

Examples of the tumor include at least one tumor selected from the group consisting of colorectal cancer, lung cancer, breast cancer, brain tumor, malignant melanoma, renal cell carcinoma, leukemia, lymphomas, gastric cancer, pancreas cancer, cervical cancer, endometrial carcinoma, ovarian cancer, esophageal cancer, liver cancer, head and neck squamous cell carcinoma, cutaneous cancer, urinary tract carcinoma, prostate cancer, choriocarcinoma, pharyngeal cancer, laryngeal cancer, thecomatosis, androblastoma, endometrium hyperplasy, endometriosis, embryoma, fibrosarcoma, Kaposi's sarcoma, hemangioma, cavernous hemangioma, angioblastoma, retinoblastoma, astrocytoma, neurofibroma, oligodendroglioma, medulloblastoma, ganglioneuroblastoma, rhabdomyosarcoma, hamartoblastoma, osteogenic sarcoma, leiomyosarcoma, thyroid sarcoma and Wilms tumor.

The present invention is explained in detail as follows.

The anti-TRAIL-R1 and R2 monoclonal antibodies have been reported to have activity to induce apoptosis in carcinoma cells [see Griffith, T. S., et al. (1999) J. Immunol. 162, 2597-2605; see Chuntharapai, A., et al. (2001) J. Immunol. 166, 4891-4898]. However, these antibodies are derived from mice.

In addition, the cytotoxicity against normal human hepatocytes, which is also questioned in a recombinant human TRAIL protein, is a concern.

Surprisingly, the novel human anti-TRAIL-R2 monoclonal antibody of the present invention has been revealed to have no side effect of inducing cytotoxicity against not only cells derived from a normal human tissue, but also normal hepatocytes for which cytotoxicity by the recombinant human TRAIL protein is a concern. We have obtained a novel anti-TRAIL-R2 monoclonal antibody which is specific to the TRAIL-R2. That is, we have completed the present invention by succeeding for the first time in the world in producing a novel monoclonal antibody provided with possible advantages of improved safety and therapeutic effects. The monoclonal antibody is preferably a whole human antibody. Its antigenicity, which is always a problem in the case of a mouse-derived antibody, has already been avoided.

Any antibody type of immunoglobulin G(IgG), A(IgA), E(IgE) or M(IgM) can be appropriately used as the antibody. Normally, IgG is more preferred.

The present invention is explained in detail by making clear the meanings of the words and phrases used in the present invention as follows.

1. TRAIL and the antibody

The antibody of the present invention is an antibody against the receptor of a tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). The antibodies of the present invention are (1) an antibody reacting with TRAIL-R1, (2) an antibody reacting with TRAIL-R2, and (3) an antibody

reacting with both TRAIL-R1 and TRAIL-R2. In the present invention, the antibody (1) may be referred to as "the anti-TRAIL-R1 antibody" and the antibodies (2) and (3) may be referred to as "the anti-TRAIL-R2 antibodies." In addition, when both TRAIL receptors, TRAIL-R1 and TRAIL-R2, are conveniently explained together in this specification, they may be referred to as "TRAIL-R1 and R2." Therefore, for example, the description of "TRAIL-R1 and R2 expression vectors" (see Example 1, below) is meant to explain two expression vectors, the expression vector of TRAIL-R1 and the expression vector of TRAIL-R2.

The "antibody" in the present invention is an antibody or a part thereof having reactivity to the human TRAIL-R1 and R2 or a part thereof as defined above, and includes functional fragments of these antibodies. The "functional fragment" means a part (partial fragment) of the antibody retaining one or more actions of the antibody on an antigen. Specific examples of functional fragments include $F(ab')_2$, Fab', Fab, Fv, disulfide-bound Fv, single chain Fv(scFv) and the polymers thereof (D. J. King., Applications and Engineering of Monoclonal Antibodies.,1998 T. J. International Ltd).

The "human antibody" in the present invention means an antibody which is the expression product of a human-derived antibody gene.

Examples of the antibody of the present invention include various antibodies having a property of inducing apoptosis in carcinoma cells expressing the human TRAIL-R1 and R2 as later described in Example 7.

The antibody of the present invention encompasses a monoclonal antibody comprising heavy chains and/or light chains having amino acid sequences with deletion, substitution or addition of one or a plurality of amino acids in each amino acid sequence of the heavy chain and/or light chain of the antibody. The above-described partial amino acid alteration (deletion, substitution, insertion or addition) can be introduced into the amino acid sequence of the antibody of the present invention by, for example, a method

which involves partial alteration of the nucleotide sequence encoding the amino acid sequence. The partial alteration can be introduced into the nucleotide sequence by a standard method using known site-specific mutagenesis (Proc Natl Acad Sci USA., 1984 Vol 81: 5662). Here, the antibody is an immunoglobulin wherein all the regions, including a heavy chain variable region and a heavy chain constant region, and a light chain variable region and a light chain constant region composing the immunoglobulin, are derived from a gene encoding the immunoglobulin.

The antibody of the present invention also encompasses antibodies having any immunoglobulin classes and isotypes.

The anti-TRAIL-R1 and R2 antibodies of the present invention can be produced by the following production method. Specifically, for example, the above-defined human TRAIL-R1 and R2 or a part thereof is bound to an appropriate substance (e.g., bovine serum albumin) for enhancing the antigenicity of an antigen, and then non-human mammals including human antibody-producing transgenic mice and the like are immunized with the bound product, together with an immunopotentiator (e.g., Freund's complete or incomplete adjuvant) if necessary. Alternatively, immunization can also be performed by introducing a gene encoding the human TRAIL-R1 or human TRAIL-R2, and then administering animal cells excessively expressing TRAIL-R1 or TRAIL-R2 on the cell surfaces. Monoclonal antibodies can be obtained by culturing hybridomas that are obtained by fusing antibody-producing cells obtained from immunized animals with myeloma cells incapable of producing autoantibodies, and then selecting clones that produce monoclonal antibodies showing specific affinity for the antigens used for immunization.

The antibody of the present invention encompasses an antibody converted to have a different subclass by alteration using genetic engineering techniques known to a person skilled in the art. For example, the subclass

switching of the antibody of the present invention to IgG2 or IgG4 enables antibodies with a low binding activity to Fc receptors to be obtained. Also, the subclass switching of the antibody of the present invention to IgG1 or IgG3 enables antibodies with a high binding activity to Fc receptors to be obtained. Moreover, the binding activity to a Fc receptor can also be changed by artificially altering the amino acid sequence of the constant region of the antibody of the present invention, or by binding with a constant region sequence having such an altered sequence. Furthermore, the therapeutic effect against diseases such as cancer can be further enhanced by binding to the antibody of the present invention a radionuclide such as iodine, yttrium, indium or technitium, (J. W. Goding, Monoclonal Antibodies: principles and practice., 1993 Academic Press), bacterial toxin such as pyocyanic toxin, diphteria toxin or lysin, chemotherapeutics such as methotrexate, mitomycin or calicheamicin (D. J. King, Applications and Engineering of Monoclonal Antibodies., 1998 T. J. International Ltd.; M. L. Grossbard., Monoclonal Antibody-Based Therapy of Cancer., 1998 Marcel Dekker Inc), or else a prodrug such as Maytansinoid (Chari et al., Cancer Res., 1992 Vol. 52: 127; Liu et al., Proc. Natl. Acad. Sci. USA, 1996 Vol. 93: 8681).

Moreover, we have found that the antibodies of the present invention having the property of binding to TRAIL-R2 but not the property of binding to TRAIL-R1 include antibodies that do not induce human hepatocyte toxicity. Therefore, the present invention also provides a method for producing anti-TRAIL-R2 antibodies having no hepatocyte toxicity, comprising a step of selecting antibodies that do not bind to TRAIL-R1 from the antibody population that binds to TRAIL-R2. However, the antibody of the present invention having no hepatocyte toxicity is not limited to an antibody having the property of binding to TRAIL-R2 but not the property of binding to TRAIL-R1.

The present invention encompasses the following operation steps in monoclonal antibody production. Specifically, the steps are, for example: (1)

purification of biopolymers and the preparation of cells excessively expressing antigen proteins on the cell surfaces (these biopolymers and/or cells are used as immunogens); (2) immunization of animals by the injection of an antigen, blood collection, testing of the antibody titer, and determination of a time for excising the spleen and the like followed by preparation of antibody-producing cells; (3) preparation of myeloma cells (hereinafter referred to as "myeloma"); (4) cell fusion of the antibody-producing cells with myeloma, (5) selection of a hybridoma group producing a target antibody; (6) division into a single cell clone (cloning); (7) if necessary, culture of hybridomas for producing monoclonal antibodies in large quantities, or breeding of animals having the hybridomas transplanted therein; and (8) study of the physiological activities and the recognition specificity of the thus-produced monoclonal antibodies, or testing of the characteristics as a labeled reagent.

The production method of anti-TRAIL-R1 and R2 monoclonal antibodies is described in detail according to the above steps, but the production method of the antibody is not limited to this method. For example, antibody-producing cells and myeloma other than splenocytes can also be used.

(1) Purification of antigen

As the antigen, a fusion protein of the extracellular regions of human TRAIL-R1 and R2 with the Fc region of a human IgG (hereinafter referred to as TRAIL-R1-hFc and TRAIL-R2-hFc) can be used. TRAIL-R1-hFc and TRAIL-R2-hFc can be obtained by integrating a DNA encoding a fusion protein of TRAIL-R1 or R2 with the Fc region of a human IgG into an expression vector for animal cells, and then purifying from the culture supernatant of the obtained transfectant strain. Alternatively, TRAIL-R1-hFc and TRAIL-R2-hFc commercially available from ALEXIS and the like can also be used. Furthermore, purified TRAIL-R1 and R2 from the cell membranes of a human cell line, can also be used as the antigen. Furthermore, the primary structures

of TRAIL-R1 and R2 are known [see Pan, G., et al. (1997) Science 276, 111-113 and Science 277, 815-818]. Thus, according to a method known by a person skilled in the art, peptides are chemically synthesized from the amino acid sequences of TRAIL-R1 and R2, and then can also be used as the antigen.

As the immunogen, Cells which are transfected with the expression vectors pEF-TRAIL-R1delta and pEF-TRAIL-R2delta, which contain a DNA encoding human TRAIL-R1 and R2 deleting the death domain and the amino acids on the C-terminal side from the death domain in the intracellular region (hereinafter referred to as "TRAIL-R1 and R2delta"), into L929 cells and excessively express TRAIL-R1 and R2delta on the cell surfaces are effective. . pEF-TRAIL-R1delta and pEF-TRAIL-R2delta can be prepared by respectively integrating a DNA encoding a human TRAIL-R1delta protein and a DNA encoding a human TRAIL-R2delta protein into pEFneo, expression vectors for animal cells. The DNAs encoding TRAIL-R1 and R2, vector, host and the like are not limited thereto.

Specifically, the transfectant strain obtained by transfecting L929 cells with pEF-TRAIL-R1 and R2delta is cultured. Using as indicators the neomycin resistance trait acquired by the cells having pEF vectors inserted therein and the confirmation of the expression of TRAIL-R1 and R2delta using goat anti-TRAIL-R1 and R2 polyclonal antibodies (DAKO), L929 cells excessively expressing human TRAIL-R1 and R2delta on the cell surfaces can be prepared.

(2) Preparation step of antibody-producing cell

The antigen obtained in (1), Freund's complete or incomplete adjuvant or an assistant such as potassium aluminum sulfate are mixed, and then experimental animals are immunized with the mixture as an immunogen. Transgenic mice capable of producing human-derived antibodies are most preferably used as experimental animals, and such mice are described in the

publication of Tomizuka et al [Tomizuka. et al., Proc Natl Acad Sci USA., 2000 Vol 97: 722].

The method for administering immunogens upon mouse immunization may be any of subcutaneous injection, intraperitoneal injection, intravenous injection, intracutaneous injection, intramuscular injection or footpad injection. Intraperitoneal injection, footpad injection or intravenous injection is preferred.

Immunization can be performed once, or repeatedly (multiple times) at appropriate intervals (intervals of preferably 3 days to 1 week). Subsequently, the antibody titer against the antigen in the serum of the immunized animal is measured, and the animals showing sufficiently increased antibody titers are used as a source of antibody-producing cells, so that the effect of the following steps can be enhanced. Generally, antibody-producing cells derived from animals 3 to 5 days after the final immunization are preferably used for the following cell fusion step.

Examples of the method for measuring antibody titer that is used herein include various known techniques such as the radioimmunoassay (hereinafter referred to as "RIA method"), enzyme-linked immunosorbent assay (hereinafter, referred to as "ELISA method"), fluorescent antibody method and passive haemagglutination method. In view of, for example, detection sensitivity, promptness, correctness, and possibility of automation of the operation, the RIA method or the ELISA method is more preferred.

In the present invention, antibody titer can be measured by the following procedures according to, for example, the ELISA method. First, purified or partially purified recombinant human TRAIL-R1 and R2 are adsorbed on the surface of a solid phase such as a 96-well plate for ELISA. The solid phase surface, on which no antigen is adsorbed, is further coated with a protein, which is independent of the antigen, such as bovine serum albumin (hereinafter referred to as "BSA"). After the surface is washed, it is allowed to come into contact with a sample (e.g., mouse serum) that has been subjected to serial

dilution as a primary antibody. Anti-TRAIL-R1 and R2 antibodies in the sample are bound to the above antigen. As a secondary antibody, enzyme-labeled antibodies against human antibodies are added and bound to the human antibodies. After washing, the substrate of the enzyme is added, and then changes and the like in absorbance due to color development resulting from substrate degradation are measured. By this method, antibody titer is calculated.

(3) Preparation step of myeloma

As myeloma, cells incapable of producing autoantibodies and derived from mammals such as mice, rats, guinea pigs, hamsters, rabbits or humans can be used. In general, established cell lines obtained from mice, for example, 8-azaguanine-resistant mouse (derived from BALB/c) myeloma strains P3X63Ag8U.1 (P3-U1) [Yelton, D.E. et al. Current Topics in Microbiology and Immunology, 81, 1-7 (1978)], P3/NSI/1-Ag4-1(NS-1) [Kohler, G. et al. European J. Immunology, 6, 511-519 (1976)], Sp2/O-Ag14(SP-2) [Shulman, M. et al. Nature, 276, 269-270 (1978)], P3X63Ag8.653 (653) [Kearney, J. F. et al. J. Immunology, 123, 1548-1550 (1979)] and P3X63Ag8 (X63) [Horibata, K. and Harris, A. W. Nature, 256, 495-497 (1975)] are preferably used. These cell lines are sub-cultured in, for example, a 8-azaguanine medium [the medium prepared by adding 8-azaguanine to an RPMI-1640 medium supplemented with glutamine, 2-mercaptoethanol, gentamicin and fetal calf serum (hereinafter referred to as "FCS")], Iscove's Modified Dulbecco's Medium (hereinafter referred to as "IMDM") or Dulbecco's Modified Eagle Medium (hereinafter referred to as "DMEM"). Subculture is performed using a normal medium 3 to 4 days before cell fusion (e.g., DMEM medium containing 10% FCS), and 2×10^7 or more cells are ensured at the day of cell fusion.

(4) Cell fusion

Antibody-producing cells are plasma cells, or lymphocytes that are progenitor cells thereof, and may be obtained from any site of an individual. In general, the cells can be obtained from, for example, the spleen, lymph node, bone marrow, tonsil, peripheral blood or an appropriate combination thereof. Splenocytes are most generally used.

After the final immunization, for example, the spleen, which is a site where antibody-producing cells are present, is excised from the mouse from which a given antibody titer is obtained, thereby preparing splenocytes, the antibody-producing cells. Currently, the most generally employed means for fusing the splenocytes with the myeloma obtained in step (3) is a method using polyethylene glycol, which has a relatively low cytotoxicity and with which the fusion procedure is simple. For example, this method comprises the following steps.

Splenocytes and myeloma are washed well in a serum-free medium (e.g., DMEM) or a phosphate-buffered saline (hereinafter referred to as "PBS"), and then mixed well to have a cell number ratio of splenocytes to myeloma of approximately 5:1 to 10:1, followed by centrifugation. The supernatant is removed, and then the precipitated cell groups are well disassembled. 1 ml of a 50% (w/v) polyethylene glycol (molecular weight of 1000 to 4000)-containing serum-free medium is dropped onto the precipitate while stirring. Subsequently, 10 ml of a serum-free medium is slowly added, and then centrifugation is performed. The supernatant is discarded again. The precipitated cells are suspended in a normal medium containing an appropriate amount of hypoxanthine, aminopterin, thymidine (hereinafter referred to as "HAT") solution (hereinafter referred to as "HAT medium") and human interleukin-6 (hereinafter referred to as "IL-6"), added in each well of a plate for culturing (hereinafter referred to as "plate"), and then cultured in the presence of 5% carbon dioxide gas at 37°C for approximately 2 weeks. Supplementation with a HAT medium is appropriately performed during culturing.

(5) Selection of hybridoma group

When the above myeloma cells are cells of an 8-azaguanine resistant strain, that is, the cells of a hypoxanthine guanine phosphoribosyltransferase (HGPRT)-deficient strain, unfused myeloma cells and myeloma-myeloma fusion cells are unable to survive in a HAT-containing medium. While a fusion cell of two antibody-producing cells, or a hybridoma of an antibody-producing cell and a myeloma cell can survive, the fusion cell of two antibody-producing cells has a limited life span. Thus, when culturing in a HAT-containing medium is continued, only hybridomas of antibody-producing cells and myeloma cells survive, so that the hybridoma can be selected.

For hybridomas grown to form colonies, the HAT medium is exchanged with a medium from which aminopterin has been removed (hereinafter referred to as "HT medium"). Subsequently, a part of the culture supernatant is collected, and then, for example, anti-TRAIL-R1 and R2 antibody titers are measured by the ELISA method. However, when the above fusion protein is used as an antigen for ELISA, a step of removing clones producing antibodies that specifically bind to the Fc region of human IgG is required so as not to select such a clone. The presence or absence of such a clone can be confirmed by, for example, ELISA using the Fc region of human IgG as an antigen.

The method using the 8-azaguanine resistant cell strain is as illustrated above. Other cell strains can also be used depending on a selection method for hybridomas. In this case, a medium composition to be used varies depending on the method used.

(6) Cloning step

Hybridomas that have been shown to produce specific antibodies by measuring antibody titer in a manner similar to that described in (2) are transferred to another plate and then subjected to cloning. Examples of the

cloning method include the limiting dilution method wherein dilution is performed to cause each well of a plate to contain one hybridoma, followed by culturing; the soft agar method, wherein culturing is performed in a soft agar medium and then colonies are collected; a method wherein each cell is picked with a micromanipulator and then the cell is cultured; and the sorter clone method, wherein one cell is separated with a cell sorter. The limiting dilution method is convenient, and is often used.

For the wells in which antibody titer has been detected, for example, cloning is repeated 2 to 4 times by the limiting dilution method, and then strains that have stable antibody titers are selected as anti-TRAIL-R1 and R2 monoclonal antibody-producing hybridoma strains.

In addition, a mouse-mouse hybridoma H-48-2 which is the human anti-TRAIL-R2 monoclonal antibody-producing cell of the present invention, was internationally deposited at the National Institute of Advanced Industrial Science and Technology (1-1-1, Higashi, Tsukuba, Ibaraki, Japan) on May 18, 2001. The international accession number is FERM BP-7599. Hence, for example, when antibodies are prepared using the mouse-mouse hybridomas, the antibodies can be prepared by step (7) and the following steps (described below) while omitting steps (1) to (6). Moreover, culturing is performed in vivo, for example, in mouse ascites, and then antibodies can be isolated from the ascites.

(7) Preparation of monoclonal antibody by culturing hybridoma

After the completion of cloning, the hybridoma is cultured in a normal medium to which HT medium is exchanged.

. Mass culture is performed by the roll-streak system using a large culture bottle, or by the spinner culture method. The supernatant in the mass culture is purified using a method known by a person skilled in the art such as gel filtration, so that anti-TRAIL-R1 and R2 monoclonal antibodies which are contained in the prophylactic or the therapeutic agent of the present invention as

an active ingredient can be obtained. Furthermore, proliferation of the hybridoma intraperitoneally in, for example, mice of the same line (e.g., BALB/c) or Nu/Nu mice, rats, guinea pigs, hamsters or rabbits makes it possible to obtain ascites containing a large amount of anti-TRAIL-R1 and R2 monoclonal antibodies which are contained in the prophylactic or the therapeutic agent of the present invention as an active ingredient. As a convenient purification method, for example, a commercially available monoclonal antibody purification kit (e.g., MAbTrap GII kit; Amersham Pharmacia Biotech) can also be used.

Monoclonal antibodies thus obtained have high antigen specificity against the human TRAIL-R1 and R2.

(8) Verification of monoclonal antibody

The isotype and the subclass of the thus-obtained monoclonal antibody can be determined as follows. Examples of identification method include the Ouchterlony method, the ELISA method and the RIA method. Although the Ouchterlony method is convenient, an enrichment step is required when the concentration of monoclonal antibodies is low.

In contrast, when the ELISA method or the RIA method is used, the culture supernatant is allowed to react intact with an antigen-coated solid phase. By further using antibodies to various immunoglobulin isotypes and subclasses as secondary antibodies, the isotype and the subclass of the monoclonal antibody can be identified.

Furthermore, protein quantification can be performed by the Folin-Lowry method, and a calculation method using absorbance at 280 nm [1.4(OD280) = immunoglobulin 1 mg/ml].

Epitopes to be recognized by monoclonal antibodies can be identified as follows. First, various partial structures of a molecule that the monoclonal antibody recognizes are prepared. To prepare the partial structures, for

example, there exist a method whereby various partial peptides of the molecule are produced using a known oligopeptide synthesis technique and a method whereby DNA sequences encoding target partial peptides are integrated into appropriate expression plasmids using genetic engineering techniques, and then the peptides are produced inside and outside a host such as *Escherichia coli*. In general, both methods are used in combination for the above purpose. For example, a series of polypeptides are prepared to be appropriately shorter in length sequentially from the C-terminus or the N-terminus of an antigen protein, using a genetic engineering technique known to a person skilled in the art. Then, the reactivities of the monoclonal antibody against them are studied, so that the approximate recognition site is determined.

Next, more specifically, various oligopeptides corresponding to the site, mutants or the like of the peptides are synthesized using an oligopeptide synthesis technique known to a person skilled in the art. Then, the ability of the monoclonal antibody (contained as an active ingredient in the prophylactic or the therapeutic agent of the present invention) to bind to these peptides is examined, or the activity of competitive inhibition of the peptide on the binding of the monoclonal antibody with the antigen is examined, thereby specifying the epitope. As a convenient method for obtaining various oligopeptides, a commercially available kit (e.g., SPOTs kit, GENOSYS BIOTECHNOLOGIES), a kit for a series of multipin peptide synthesis (Chiron) using the multipin syntheses method or the like can also be used.

Moreover, a gene encoding a human monoclonal antibody is cloned from an antibody-producing cell such as a hybridoma, the gene is integrated into an appropriate vector, and then the vector is introduced into a host (e.g., a mammalian cell line, *Escherichia coli*, yeast cells, insect cells or plant cells). Thus, recombinant antibodies that are produced using the gene recombinant technique can be prepared (P. J. Delves., ANTIBODY PRODUCTION ESSENTIAL TECHNIQUES., 1997 WILEY, P. Shepherd and C. Dean.,

Monoclonal Antibodies., 2000 OXFORD UNIVERSITY PRESS, J. W. Goding., Monoclonal Antibodies: principles and practice., 1993 ACADEMIC PRESS).

Moreover, by the use of transgenic animal generation techniques, transgenic cattle, transgenic goats, transgenic sheep or transgenic pigs having the gene of a target antibody integrated in the endogenous gene are generated. The antibody gene-derived monoclonal antibodies can then be obtained in large quantities from the milk to be secreted from the transgenic animal. Hybridomas can be cultured in vitro using a known nutrition medium, which is used to allow the proliferation, maintenance, and storage of the hybridoma so as to cause the hybridoma to produce monoclonal antibodies in the culture supernatant depending on various conditions such as the characteristics of a cell type to be cultured, the purpose of an experiment or study, and a culture method; or any nutrition medium, which is induced and prepared from a known basic medium.

The antibody of the present invention has the following functional properties (a) to (c), and each of the properties can be confirmed by, for example, the method described for each of (a) to (c).

(a) When human carcinoma cells are cultured, the antibody of the present invention is contained in the medium, and the survival rate of the cells is examined, the antibody has activity to induce apoptosis in carcinoma cells expressing TRAIL-R1 and/or R2.

(b) When normal human tissue-derived cells are cultured, the antibody of the present invention is contained in the medium, and the survival rate of the cells is examined, the antibody does not have effect on normal cells (except for hepatic parenchymal cells) expressing TRAIL-R1 and/or R2.

(c) When human hepatocytes are cultured, the antibody of the present invention is contained in the medium, and the survival rate of the cells is examined, the antibody does not induce hepatocyte toxicity.

The antibody of the present invention has any of the above activities (a) to (c). The antibody is a substance having novel characteristics in that it preferably has the above activity (a) of inducing apoptosis in carcinoma cells, and the above activities (b) and (c) of not inducing damage on normal cells, particularly normal hepatocytes. Therefore, the antibody of the present invention is useful as an ingredient to be contained in a prophylactic or therapeutic agent against malignant tumors.

It can be confirmed that the antibody of the present invention has the activity of inducing apoptosis by culturing cells (e.g., human colon cancer cell line Colo205 (American Type Culture Collection No. CCL-222)) in a media added with a sample and measuring the viability of the cells by a method such as MTT assay (See Green, L. M., et al. (1984) J. Immunological Methods 70, 257-268).

Pharmaceutical composition

A preparation containing a preparation that is prepared by purifying the human anti-TRAIL-R1 and R2 antibodies of the present invention is also encompassed within the scope of the present invention. Such a preparation preferably contains a physiologically acceptable diluent or carrier in addition to the antibody, and may be a mixture with other antibodies or other drugs such as antibiotics. Examples of an appropriate carrier include, but are not limited to, a physiological saline solution, a phosphate buffered saline solution, a phosphate buffered saline glucose solution and a buffered physiological saline. Alternatively, the antibody may be freeze-dried, and then used when necessary by adding the above buffered aqueous solution for reconstitution. The prophylactic or therapeutic agent can be administered in various forms. Examples of the forms of administration of these agents include oral administration using vehicles such as tablets, capsules, granules, powders or syrups, and parenteral administration using vehicles such as injections, drops or

suppositories.

The dose differs depending on symptom, age, body weight and the like. Normally in the case of oral administration, the dose is approximately 0.01 mg to 1000 mg per day for an adult, and it can be administered once or separately administered on several different occasions. Further, in the case of parenteral administration, a dose of approximately 0.1 mg to 1000 mg per administration can be administered by subcutaneous injection, intramuscular injection or intravenous injection.

The antibody or the pharmaceutical composition of the present invention can be applied to treatment of or prophylaxis against various diseases or symptoms that may be caused by cells expressing TRAIL-R1 and R2. Examples of such diseases or the symptoms include various malignant tumors.

Examples of the types of such tumors include colorectal cancer, lung cancer, breast cancer, brain tumor, malignant melanoma, renal cell carcinoma, leukemia, lymphomas, gastric cancer, pancreas cancer, cervical cancer, endometrial carcinoma, ovarian cancer, esophageal cancer, liver cancer, head and neck squamous cell carcinoma, cutaneous cancer, urinary tract carcinoma, prostate cancer, choriocarcinoma, pharyngeal cancer, laryngeal cancer, thecomatosis, androblastoma, endometrium hyperplasy, endometriosis, embryoma, fibrosarcoma, Kaposi's sarcoma, hemangioma, cavernous hemangioma, angioblastoma, retinoblastoma, astrocytoma, neurofibroma, oligodendroglioma, medulloblastoma, ganglioneuroblastoma, rhabdomyosarcoma, hamartoblastoma, osteogenic sarcoma, leiomyosarcoma, thyroid sarcoma and Wilms tumor. The number of the types of tumors to which the antibody of the present invention is applied is not limited to one type, and plural types of tumors may develop at the same time.

Example of preparation

The molecule of the present invention is used in the form of an ampule

of aseptic solution or suspension prepared by dissolving the molecule in water or a pharmacologically acceptable solution other than water. In addition, an ampule may be filled with an aseptic powder preparation (preferably, where the molecule of the present invention is freeze-dried), and it can be diluted with a pharmacologically acceptable solution when used.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the cell-death-inducing activity on Colo205 in the culture supernatant of hybridomas producing human anti-TRAIL-R1 monoclonal antibodies.

Figure 2 shows the cell-death-inducing activity on Colo205 in the culture supernatant of hybridomas producing human anti-TRAIL-R2 monoclonal antibodies.

Figure 3 shows the cell-death-inducing activity on Colo205 in the culture supernatant of hybridomas producing human anti-TRAIL-R2 monoclonal antibodies (Goat anti-human IgG(γ) specific polyclonal antibodies were not present).

Figure 4 shows the cell-death-inducing activity on HUVEC in the culture supernatant of hybridomas producing human anti-TRAIL-R2 monoclonal antibodies.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention will be described more specifically by the following examples. The present invention is not limited to the embodiments described in these examples.

Example 1 Preparation of antigen

To obtain cells excessively expressing human TRAIL-R1 and R2 on the

cell membrane, plasmid vectors for the expression of human TRAIL R1 and human TRAIL-R2 (which had been prepared by removing the death domain and the amino acids on the C-terminal side from the death domain in the intracellular regions from the full-length amino acids of the human TRAIL-R1 and R2, hereinafter referred to as TRAIL-R1 and R2delta,) were prepared. DNAs encoding TRAIL-R1 and R2delta were prepared by the PCR method.

a) Construction of full-length human TRAIL-R1 and R2 expression vectors

To perform template PCR, plasmid vectors, pcDNA3-TRAIL-R1 and pcDNA3-TRAIL-R2, retaining cDNAs encoding human TRAIL-R1 and R2 were used as templates. pcDNA3-TRAIL-R1 and pcDNA3-TRAIL-R2 were constructed by the following method. The full-length human TRAIL-R1 DNA and TRAIL-R2 DNA were modified by polymerase chain reaction (PCR) to add an *Eco*R I sequence to the 5' end, and a *Not* I sequence and a termination codon to the 3' end. Using human placenta-derived cDNA (Clontech) as a template, primers 5'-CACGAATTCACCATGGCGCCACCACCAGCT-3' (SEQ ID NO: 1) and

5'-TTTCTCGAGGCGGCCGCTTATCACTCCAAGGACACGGCAGAGCCTGT G-3' (SEQ ID NO: 2) synthesized for TRAIL-R1, and primers 5'-CACGAATTCGCCACCATGGAACAACGGGGACAG-3' (SEQ ID NO: 3) and

5'-TTTCTCGAGGCGGCCGCTCATTAGGACATGGCAGAGTCTGCATTACCT -3' (SEQ ID NO: 4) synthesized for TRAIL-R2, a PCR reaction was performed for 30 cycles (each cycle consisting of 94°C for 20 seconds, 60°C for 30 seconds and 68°C for 90 seconds) using platinum PfxDNA polymerase (Gibco BRL). The modified TRAIL-R1 and TRAIL-R2 sequences were isolated as *Eco*R I-*Not* I fragments, and then ligated to pcDNA3 (Invitrogen) vectors that had been cleaved with the same enzymes. The obtained plasmids were named pcDNA3-TRAIL-R1 and pcDNA3-TRAIL-R2. Hereinafter, the reaction

temperature for all the PCRs in the examples was regulated using a GeneAmp PCR system 9700 (Perkin Elmer Japan).

b) Construction of human TRAIL-R1 and R2delta expression vectors

Human TRAIL-R1 and R2delta expression vectors were constructed by the following methods. To prepare an expression plasmid comprising a TRAIL-R1 partial peptide having an amino acid sequence of 1 to 351, and the same comprising a TRAIL-R2 partial peptide having an amino acid sequence of 1 to 348, PCR reaction was performed to add an *Eco*R I sequence to the 5' ends of the TRAIL-R1 and R2 partial peptides, and an *Not* I sequence and a termination codon to the 3' ends of the same. PCR was performed for 25 cycles (each cycle consisting of 94°C for 20 seconds, 65°C for 30 seconds and 68°C for 75 seconds) using oligonucleotide primers 5'-CACGAATTCACCATGGCGCCACCACCAGCT-3' (SEQ ID NO: 1) and 5'-TTCTACGAGCGGCTTATCACAGCCTCCTCCTCTGAGA-3' (SEQ ID NO: 5) for TRAIL-R1, and oligonulcotide primers 5'-CACGAATTCGCCACCATGGAACAACGGGGACAG-3' (SEQ ID NO: 3) and 5'-TTCTACGAGCGGCCGCTTATCACAAGTCTGCAAAGTCATC-3' (SEQ ID NO: 6) for TRAIL-R2, platinum PfxDNA polymerase (Gibco BRL), pcDNA3-TRAIL-R1 and pcDNA3-TRAIL-R2. The modified TRAIL-R1 and R2 partial peptides were isolated as *Eco*R I-*Not* I fragments. The *Eco*R I-*Not* I fragment was ligated to pEFneo vectors (an expression vector having EF promoter) that had been cleaved with *Eco*R I and *Not* I enzymes. The obtained plasmids were named pEF-TRAIL-R1delta and pEF-TRAIL-R2delta.

c) Preparation of human TRAIL-R1 and R2delta-expressing cells

pEF-TRAIL-R1delta and pEF-TRAIL-R2delta prepared in b) were introduced into L929 cells (American Type Culture Collection No.CCL-1) using LipofectAMINE Plus (Gibco BRL). Transfection was performed by the

method described in the manual. After 24 hours of culturing in a flask for culturing cells (with a culture area of 75 cm^2) at 37°C under 5.0% carbon dioxide gas, G418 (Gibco BRL) was added at 1 mg/ml in the culture, followed by 1 week of culturing. Subsequently, FACS analysis was performed using goat anti-human TRAIL-R1 polyclonal antibodies and goat anti-human TRAIL-R2 polyclonal antibodies (DAKO). Thus, it was confirmed that the transfected cells, which had acquired a G418 resistance trait, expressed TRAIL-R1delta comprising 351 amino acids and TRAIL-R2delta on the cell membrane surface.

The synthesis of oligonucleotides such as primers for PCR was always performed using an automated DNA synthesis system (model 3948, Perkin Elmer Japan, Applied Biosystems division) according to the manual [see Matteucci, M. D. and Caruthers, M.H. (1981) J. Am. Chem. Soc. 103, 3185-3191]. After the end of synthesis, each oligonucleotide was cleaved from the support and then deprotected. The obtained solution was dried and solidified, and the product was dissolved in distilled water, and then cryopreserved at -20°C until use.

Example 2 Generation of human antibody-producing mice

The mice used for immunization had a genetic background whereby they were homozygotes for both disrupted endogenous Ig heavy chain and κ light chain, and the mice harbored at the same time chromosome 14 fragment (SC20) containing a human Ig heavy chain locus, and a human Igκ chain transgene (KCo5). These mice were generated by crossing mice of a line A having a human Ig heavy chain locus with mice of a line B having a human Igκ chain transgene. The mice of line A are homozygotes for both disrupted endogenous Ig heavy chain and κ light chain, and harbor chromosome 14 fragment (SC20), which is transmittable to progeny, as is described, for example, in the report of Tomizuka et al. (Tomizuka. et al., Proc Natl Acad Sci USA., 2000 Vol 97: 722).

Furthermore, the mice of line B (transgenic mice) are homozygotes for both disrupted endogenous Ig heavy chain and κ light chain, and harbor a human Igκ chain transgene (KCo5), as described, for example, in the report of Fishwild et al. (Nat Biotechnol., 1996 Vol 14:845).

Progeny mice obtained by crossing male mice of the line A with female mice of the line B, or female mice of the line A with male mice of the line B, were analyzed by the method described in Tomizuka et al's report (Tomizuka et al., Proc Natl Acad Sci USA., 2000 Vol 97:722). Individuals (human antibody-producing mice) having human Ig heavy chain and κ light chain detected simultaneously in the sera were screened for (Ishida & Lonberg, IBC's 11th Antibody Engineering, Abstract 2000) and used for the following immunization experiment. In addition, the above human antibody-producing mice are available from Kirin Brewery Co., Ltd via contract.

Example 3 Preparation of human monoclonal antibodies against human TRAIL-R1 and R2

In this example, monoclonal antibodies were prepared according to general methods as described in, for example, Introduction of Experimental Protocols for Monoclonal Antibody (Monoclonal Antibody Jikken Sosa Nyumon, written by Tamie ANDO et al., KODANSHA, 1991). As immunogens, the TRAIL-R1 and R2delta-expressing L929 cell prepared in Example 1 was used. Animals used for immunization were the human antibody (human immunoglobulin)-producing mice generated in Example 2.

Human antibody-producing mice were initially immunized via the right foot pad with the TRAIL-R1delta-expressing L929 cells and TRAIL-R2 delta-expressing L929 cells (5×10^6 cells/mouse) prepared in Example 1. After the initial immunization, immunization with the L929 cells was performed 10 times every 3 days via the left and right food pad alternatively. Furthermore, at 3 days before the obtainment of the spleen and the lymph node (described later),

the L929 cells were used for immunization via both foot pads.

The spleens and the lymph nodes were obtained by a surgical operation from the immunized mice. Then the organ was put into 10 ml of a serum-free DMEM medium (Gibco BRL) containing 350 mg/ml sodium hydrogen carbonate, 50 units/ml penicillin and 50 μg/ml streptomycin (hereinafter, referred to as "serum-free DMEM medium"). It was then pulverized using a spatula on mesh (Cell strainer: Falcon). The cell suspension that had passed through the mesh was centrifuged so as to precipitate the cells. The cells were washed twice in a serum-free DMEM medium, and suspended in a serum-free DMEM medium, and then the number of the cells was counted. In the meantime, myeloma cells SP2/0 (ATCC No. CRL-1581) that had been cultured so as not to exceed a cell concentration of 1×10^8 cells/ml at 37°C in the presence of 5% carbon dioxide gas in a 10% FCS (Sigma)-containing DMEM medium (Gibco BRL) (hereinafter referred to as "serum-containing DMEM medium") were washed in a serum-free DMEM medium in the same manner. Then the cells were suspended in a serum-free DMEM medium, and then the number of the cells was counted. The collected cell suspension and the mouse myeloma suspension were mixed at a cell number ratio of 5:1. The mixture was centrifuged, thereby completely removing the supernatant. To this pellet, 1 ml of 50% (w/v) polyethylene glycol 1500 (Boehringer Mannheim) was gently added as a fusion agent while agitating the pellet using the tip of a pipette. Next, 1 ml of a serum-free DMEM medium preheated at 37°C was gently added at two separate times, followed by addition of another 7 ml of serum-free DMEM medium. After centrifugation, the fusion cells obtained by the removal of the supernatant were subjected to screening by the limiting dilution method described below. Screening for hybridomas was performed by culturing the cells in DMEM media containing 10% fetal calf serum (FCS), hypoxanthine (H), aminopterin (A) and thymidine (T) (hereinafter referred to as "HAT": Sigma). Further, single clones were obtained using DMEM media containing HT (Sigma) by the limiting

dilution method. Culturing was performed in a 96-well microtiter plate (Beckton Dickinson). Screening for hybridoma clones producing anti-human TRAIL-R1 and R2 human monoclonal antibodies and characterization of the human monoclonal antibodies produced by each of the hybridomas were performed by the enzyme-linked immunosorbent assay (ELISA) and fluorescence activated cell sorter (FACS) described below.

For the screening of human monoclonal antibody producing cells hybridoma by ELISA, by ELISA described in Examples 4 and 5 and the FACS analysis described in Example 6, a large number of hybridomas producing human monoclonal antibodies that have human immunoglobulin γ chain (hIg γ) and human immunoglobulin light chain κ, and have reactivity specifically to human TRAIL-R1 and/or R2 were obtained. Furthermore, in any of the following examples including this example, and tables and figures showing the test results of the examples, hybridoma clones producing each of the human anti-human TRAIL-R1 and R2 monoclonal antibodies of the present invention were denoted using symbols. The following hybridoma clones represent single clones: 1-13-6, 1-32-9, 1-40-4, 1-43-43, 2-6-48, 2-11-5, 2-12-10, 2-47-11, 2-52-12, 3-10-19, 3-23-8, 3-33-7, 3-42-3, 3-53-15, 2-18-2, 3-1-7, E-11-13, E-14-4, F-4-2, F-4-8, H-48-2, L-30-10, N-18-12, W-40-5, X-14-4, X-51-4, X-51-12, A-4-29, G-3-10, H-34-2, K-57-12 and W-42-2. H-48-2 of these clones was internationally deposited at the National Institute of Advanced Industrial Science and Technology (1-1-1, Higashi, Tsukuba, Ibaraki, Japan) on May 18, 2001.

Example 4 Detection of human anti-TRAIL-R1 monoclonal antibody or human anti-TRAIL-R2 monoclonal antibody having human immunoglobulin light chain κ (Igκ)

Fusion proteins of the extracellular regions of human TRAIL-R1 and R2

and the Fc region of human IgG1 (hereinafter referred to as "TRAIL-R1-hFc" and "TRAIL-R2-hFc" (ALEXIS)) were added at 0.5μg/ml in phosphate buffered saline (hereinafter referred to as "PBS"). 50 μl of the thus prepared solution was added to each well of a 96-well microplate for ELISA (Maxisorp, Nunc) and incubated for 30 minutes at room temperature, thereby coating TRAIL-R1-hFc or TRAIL-R2-hFc to the microplate. Subsequently the supernatant was discarded, a blocking reagent (SuperBlock (registered trademark) Blocking Buffer, PIERCE) was added to each well, and then incubation was performed at room temperature for 30 minutes, thereby blocking the part where TRAIL-R1-hFc or TRAIL-R2-hFc did not bind. Thus, a microplate having each well coated with TRAIL-R1-hFc or TRAIL-R2-hFc was prepared.

The culture supernatant of each hybridoma (50 μl) was added to each well, reaction was performed at room temperature for 30 minutes, and then each well was washed twice in 0.1% Tween20-containing PBS (PBS-T). Subsequently, horseradish peroxidase-labeled goat anti-human Igκ antibodies (50 μl/well, Biosource International) were diluted 2000 times in PBS-T containing 10% Block Ace (Dainippon Pharmaceutical Co., Ltd.). 50 μl of the thus prepared solution was added to each well, and incubation was then performed at room temperature for 30 minutes. The microplate was washed three times with PBS-T, and then 100 μl of a TMB chromogenic substrate solution (DAKO) was added to each well, followed by incubation at room temperature for 20 minutes. 0.5M sulfuric acid was added (100 μl/well) to each well to stop reaction. Absorbance at a wavelength of 450 nm (reference wavelength of 570 nm) was measured with a microplate reader (MTP-300, Corona Electric).

Table 1 and Table 2 show the part of antibodies among the thus obtained anti-human TRAIL-R1 and R2 antibodies. Table 1 shows the subclass and cross reactivity of the obtained human anti-TRAIL-R1 monoclonal antibodies. Table 2 shows the subclass and cross reactivity of the obtained human

anti-TRAIL-R2 monoclonal antibodies.

Table 1

Human anti-TRAIL-R1 antibody	Subclass	Cross reactivity	
		TRAIL-R1	TRAIL-R2
1-13	IgG4	+	−
1-18	IgG4	+	−
1-32	IgG1	+	−
1-40	IgG1	+	−
1-43	IgG1	+	−
2-6	IgG1	+	−
2-11	IgG1	+	−
2-12	IgG1	+	−
2-18	IgM	+	−
2-47	IgG4	+	−
2-52	IgG1	+	−
3-1	IgM	+	−
3-7	IgM	+	−
3-10	IgG4	+	−
3-23	IgG4	+	−
3-33	IgG4	+	−
3-42	IgG2	+	−
3-53	IgG1	+	−

+ : with reactivity

− : no reactivity

Table 2

Human anti-TRAIL-R2 antibody	Subclass	Cross reactivity	
		TRAIL-R1	TRAIL-R2
A-4-27	IgM	−	+
A-4-29	IgM	+	+
A-11	IgM	−	+
E-11	IgG1	−	+
E-14	IgG1	−	+
F-4-2	IgG4	−	+
F-4-8	IgG1	−	+
G-3	IgM	−	+
H-34	IgM	−	+
H-48-2	IgG1	−	+
I-22	IgM	−	+
I-35	IgM	−	+
J-21	IgM	−	+
J-26	IgM	−	+
K-8	IgM	−	+
K-16	IgM	−	+
K-57	IgM	−	+
L-4	IgM	−	+
L-30	IgG1	−	+
N-18	IgG4	−	+
P-28	IgM	−	+
P-36	IgM	−	+
W-40-5	IgG1	−	+
W-42	IgM	−	+
X-13	IgM	−	+

X-14	IgG4	−	+
X-51-4	IgG1	−	+
X-51-12	IgG4	−	+
X-60	IgM	−	+
Z-23	IgM	−	+
1-39	IgM	−	+

+ : with reactivity

− : no reactivity

Example 5 Identification of the subclass of each monoclonal antibody

A microplate having each well coated with TRAIL-R1-hFc or TRAIL-R2-hFc was prepared by a method similar to that of Example 4, and then each well was washed twice with PBS-T. The culture supernatant (50 μl) of each of the hybridomas obtained in Example 4 was added to each well of the microplate coated with TRAIL-R1-hFc or TRAIL-R2-hFc to perform reaction for 30 minutes, and then each well was washed twice with PBS-T. Subsequently, sheep anti-human IgG1 antibodies, sheep anti-human IgG2 antibodies, or sheep anti-human IgG3 antibodies or sheep anti-human IgG4 antibodies, which had been respectively labeled with horseradish peroxidase and diluted 2000 times, were added (50 μl/well, The Binding Site) to each well, followed by incubation at room temperature for 30 minutes. After washing 3 times with PBS-T, a substrate buffer (TMB, 100 μl/well, DAKO) was added to each well, and then incubation was performed at room temperature for 20 minutes. Next, 0.5M sulfuric acid (100 μl/well) was added to stop the reaction. Absorbance at a wave length of 450 nm (with a reference wavelength of 570 nm) was measured using a microplate reader (MTP-300, Corona Electric). The above Table 1 and Table 2 show the results.

Example 6 Test of the reactivity of each monoclonal antibody to TRAIL-R1 and R2 expressing cells

The reactivity of each of the monoclonal antibodies obtained in Example 4 to the TRAIL-R1delta-expressing L929 cells and TRAIL-R2delta-expressing L929 cells prepared in Example 1 was examined by FACS analysis. L929 cells, TRAIL-R1delta-expressing L929 cells and TRAIL-R2delta-expressing L929 cells were suspended at a concentration of $2x10^6$/ml in a staining buffer (SB) of PBS containing 1% rabbit serum, 0.1% NaN_3 and 1% FCS. The cell suspension (100 μl/well) was added into a 96-well round-bottomed plate (Beckton Dickinson). After centrifugation (2000 rpm, 4°C, 2 minutes), the

supernatant was removed and then the culture supernatant (50 μl) of the hybridoma cultured in Example 3 was added. The mixture was agitated, allowed to stand on ice for 30 minutes, and then subjected to centrifugation (2000 rpm, 4°C for 2 minutes) to remove the supernatant. After the pellet was washed twice with SB (100 μl/well), 30 μl of 0.0125 mg/ml RPE fluorescence-labeled rabbit anti-human Igκ $F(ab')_2$ antibodies (DAKO) was added, and then incubation was performed on ice for 30 minutes. After washed twice with SB, the cells were suspended in 300 μl of SB, and then fluorescence intensity of each cell was measured by FACS (FACScan, Beckton Dickinson). As a result, all the antibodies were observed to have strong binding activity only to the TRAIL-R1delta-expressing L929 cells or the TRAIL-R2delta-expressing L929 cells, and no binding activity to L929 cells was observed. Thus, it was shown that they were antibodies binding specifically to TRAIL-R1 and TRAIL-R2.

Example 7 Cell-death-inducing activity on carcinoma cells

Using the culture supernatant of the hybridoma producing the human anti-TRAIL-R1 monoclonal antibodies or the human anti-TRAIL-R2 monoclonal antibodies obtained from Example 4 to 6, cell-death-inducing activity on Colo205 (ATCC No. CCL-222) cells, which were colon carcinoma cells, was measured. Colo205 cells cultured in RPMI media containing 10% FCS were prepared at a concentration of 2.5×10^4/ml. 100 μl of the suspension was added to each well of a 96-well flat bottomed plate (Beckton Dickinson). After culturing at 37°C under 5.0% carbon dioxide gas for 24 hours, the hybridoma culture supernatant was added at 50 μl/well. Furthermore, goat anti-human IgG (γ)-specific polyclonal antibodies (Sigma) were added (10μl/well) to each well at a final concentration of 5 μg/ml. For a part of the obtained hybridomas, wells not supplemented with goat anti-human IgG (γ)-specific polyclonal antibodies were prepared. As a positive control, human

recombinant TRAIL protein (DAKO) was employed with a final concentration of 100 ng/ml. As a negative control, human IgG (Biogenesis) was employed. After 48 hours of culturing at 37°C under 5.0% carbon dioxide gas, an MTS reagent (Cell Titer 96 AQUEOUS Non-Radioactive Cell Proliferation Assay: Promega) was prepared according to the method described in the instructions, and then 20 μl of the reagent was added to each well. After another 2 hours of culturing at 37°C under 5.0% carbon dioxide gas, absorbance at a wavelength of 490 nm (reference wavelength of 630 nm) was measured using a microplate reader (1420 ARVO multi-label counter: WALLAC). Using the reducibility of mitochondria as an indicator, the survival rate of the cells was calculated. The survival rate of the cells in each well was calculated by the following formula: Survival rate (%) = 100 × (a-b)/(c-b) (wherein "a" represents the measured value of a well tested, "b" represents the measured value of a cell-free well, and "c" represents the measured value of a negative control well). Figures 1 to 3 and Tables 3 and 4 show the results. Table 3 shows the cell-death-inducing activity (in the culture supernatant of the hybridomas producing the human anti-TRAIL-R1 monoclonal antibodies) on normal human hepatocytes. Table 4 shows the cell-death-inducing activity (in the culture supernatant of the hybridomas producing the human anti-TRAIL-R2 monoclonal antibodies) on human normal heptocytes.

Table 3

Human anti-TRAIL-R1 antibody	**Subclass**	**Normal human hepatocyte survival rate**	**Colo205 cell survival rate**
1-13-6	**IgG4**	—	—
1-32-9	**IgG1**	—	—
1-40-4	**IgG1**	—	—

1-43-43	IgG1	−	−
2-6-48	IgG1	−	−
2-11-5	IgG1	++	++
2-12-10	IgG1	−	−
2-47-11	IgG4	+	+
2-52-12	IgG1	++	++
3-10-19	IgG4	−	−
3-23-8	IgG4	−	−
3-33-7	IgG4	−	−
3-42-3	IgG2	−	−
3-53-15	IgG1	−	−
2-18-2	IgM	++	++
3-1-7	IgM	−	+
sTRAIL 1 μg/ml	-	−	−

++: Survival rate of 80% or more
+: Survival rate of 21% to 79%
−: Survival rate of 20% or less

Table 4

Human anti-TRAIL-R2 antibody	Subclass	Normal human hepatocyte survival rate	Colo205 cell survival rate
E-11-13	IgG1	++	−
E-14-4	IgG1	+	+
F-4-2	IgG4	+	−
F-4-8	IgG1	−	−
H-48-2	IgG1	++	−
L-30-10	IgG1	++	−
N-18-12	IgG4	++	−
W-40-5	IgG1	++	+
X-14-4	IgG4	++	+
W-51-4	IgG1	−	−
X-51-12	IgG4	++	−
A-4-29	IgM	−	−
G-3-10	IgM	++	−
H-34-2	IgM	−	−
K-57-12	IgM	+	−
W-42-2	IgM	−	−
sTRAIL 1 μg/ml	-	−	−

++: Survival rate of 80% or more
+: Survival rate of 21% to 79%
− : Survival rate of 20% or less

As a result, it was revealed that the human anti-TRAIL-R1 and R2 monoclonal antibodies clearly had activity to induce cell death in Colo205 cells, compared with the negative control. Moreover, it was shown that a part of the human anti-TRAIL-R2 monoclonal antibodies had activity to induce cell death

even in the absence of goat anti-human IgG(γ)-specific polyclonal antibodies (in a state without cross-linking with the human anti-TRAIL-R2 monoclonal antibodies).

Example 8 Cell-death-inducing activity on normal cells

Cell-death-inducing activity on HUVEC (Biowhittaker), which is a normal human umbilical vein endothelial cell, was measured using the culture supernatant of the hybridomas producing the human anti-TRAIL-R2 monoclonal antibodies obtained in Examples 4 to 6. HUVEC cells cultured in an EGM-2 medium (Biowhittaker) were prepared at a concentration of 5×10^4/ml. 100 μl of the suspension was added to each well of a 96-well flat-bottomed plate (Beckton Dickinson). The cells were cultured at 37°C under 5.0% carbon dioxide gas for 24 hours, and then the culture supernatant of the hybridoma was added at 50 μl/well. Further, 10 μl of goat anti-human IgG(γ)-specific polyclonal antibodies (Sigma) were added at a final concentration of 5 μg/ml to each well. Human IgG (Biogenesis) was used as a negative control. After 48 hours of culturing at 37°C under 5.0% carbon dioxide gas, an MTS reagent (Cell Titer 96 AQ$_{UEOUS}$ Non-Radioactive Cell Proliferation Assay: Promega) was prepared according to the method described in the instructions, and then 20μl of the reagent was added to each well. After another 2 hours of culturing at 37°C under 5.0% carbon dioxide gas, absorbance at a wavelength of 490 nm (with a reference wavelength of 630 nm) was measured using a microplate reader (1420 ARVO multi-label counter: WALLAC). Using the reducibility of mitochondria as an indicator, the survival rate of the cells was calculated. The survival rate of the cells of each well was calculated by a formula similar to that of Example 7.

Figure 4 shows the result. The human anti-TRAIL-R2 monoclonal antibody and the negative control showed almost the same result, revealing that the human anti-TRAIL-R2 monoclonal antibody does not show cytotoxicity

against HUVEC cells.

Example 9 Cell-death-inducing activity on normal human hepatocytes

Cell-death-inducing activity on normal human hepatocytes (hereinafter referred to as "HH cells") (K.A.C.) was measured using the culture supernatant of the hybridomas producing the human anti-TRAIL-R1 and R2 monoclonal antibodies obtained in Examples 4 to 6. First, frozen HH cells were thawed at 37°C, and then prepared at a concentration of 7.5×10^5/ml using a CM5300 medium (K.A.C.). 100 μl of the suspension was added to each well of a 96-well flat-bottomed plate coated with collagen type I (Beckton Dickinson). After 4.5 hours of culturing at 37°C under 5.0% carbon dioxide gas, medium exchange was performed. After 24 hours of culturing at 37°C under 5.0% carbon dioxide gas, medium exchange was performed again. Subsequently, the culture supernatant of the hybridoma was added at 50 μl/well, and then 10 μl of goat anti-human IgG(γ)-specific polyclonal antibodies (Sigma) were added to each well at a final concentration of 5 μg/ml. Human IgG (Biogenesis) was used as a negative control. After 24 hours of culturing at 37°C under 5.0% carbon dioxide gas, morphological changes in HH cells were observed under a microscope. The result of the human anti-TRAIL-R2 monoclonal antibody and that of negative control were almost the same, revealing that the human anti-TRAIL-R2 monoclonal antibody does not show cytotoxicity also against HH cells.

Industrial Applicability

According to the present invention, there is provided a molecule with extremely high safety, which is useful as a prophylactic or therapeutic agent against disease, in particular malignant tumors, caused by TRAIL-R1 and R2-expressing cells, and which can avoid damage to the liver.

Sequence Listing Free Text

SEQ ID NO: 1: synthetic DNA

SEQ ID NO: 2: synthetic DNA

SEQ ID NO: 3: synthetic DNA

SEQ ID NO: 4: synthetic DNA

SEQ ID NO: 5: synthetic DNA

SEQ ID NO: 6: synthetic DNA

CLAIMS

1. An antibody or a functional fragment thereof, binding to TRAIL-R1 and/or TRAIL-R2.

2. The antibody or the functional fragment thereof of claim 1, having at least one property selected from the following (a) to (c) of:

(a) having activity to induce apoptosis in carcinoma cells expressing TRAIL-R1 and/or TRAIL-R2;

(b) not having effect on normal human cells (except for hepatic parenchymal cells) expressing TRAIL-R1 and/or TRAIL-R2; and

(c) not inducing human hepatocyte toxicity.

3. An antibody or a functional fragment thereof, having all the following properties (a) to (c) of:

(a) having activity to induce apoptosis in carcinoma cells expressing TRAIL-R1 and/or TRAIL-R2;

(b) not having effect on normal human cells (except for hepatic parenchymal cells) expressing TRAIL-R1 and/or TRAIL-R2; and

(c) not inducing human hepatocyte toxicity.

4. The antibody or the functional fragment thereof of claim 2 or 3, which binds to TRAIL-R2, but does not bind to TRAIL-R1.

5. The antibody or the functional fragment thereof of any one of claims 1 to 4, which is a monoclonal antibody produced by a mouse-mouse hybridoma.

6. The antibody or the functional fragment thereof of any one of claims 1 to 5, which is a human antibody.

7. The antibody or the functional fragment thereof of any one of claims 1 to 6, which is an immunoglobulin G antibody.

8. An antibody or a functional fragment thereof binding to TRAIL-R1 or TRAIL-R2, which is produced by a hybridoma E-11-13, H-48-2, L-30-10, N-18-12, W-40-5, X-14-4, X-51-12, or G-3-10.

9. An antibody or a functional fragment thereof binding to TRAIL-R1 or TRAIL-R2, which is produced by a hybridoma H-48-2 with the accession number of FERM BP-7599.

10. A hybridoma producing monoclonal antibodies that bind to TRAIL-R2, which is selected from the group consisting of E-11-13, H-48-2, L-30-10, N-18-12, W-40-5, X-14-4, X-51-12, and G-3-10.

11. A hybridoma H-48-2 producing monoclonal antibodies that bind to TRAIL-R2, with the accession number of FERM BP-7599.

12. A method for producing anti-TRAIL-R2 monoclonal antibodies, comprising culturing the hybridoma of claim 10 or 11, and collecting the antibodies binding to TRAIL-R2 from the obtained culture product.

13. A method for producing anti-TRAIL-R2 antibody which does not have hepatocyte toxicity, comprising selecting an antibody which does not bind to TRAIL-R1 from the population of antibodies which bind to TRAIL-R2.

14. A prophylactic or therapeutic agent against tumors, comprising as an active ingredient the antibody or the functional fragment thereof of any one of claims 1 to 9.

15. The prophylactic or therapeutic agent of claim 14, wherein the tumor is any one tumor selected from the group consisting of colorectal cancer, lung cancer, breast cancer, brain tumor, malignant melanoma, renal cell carcinoma, leukemia, lymphomas, gastric cancer, pancreas cancer, cervical cancer, endometrial carcinoma, ovarian cancer, esophageal cancer, liver cancer, head and neck squamous cell carcinoma, cutaneous cancer, urinary tract carcinoma, prostate cancer, choriocarcinoma, pharyngeal cancer, laryngeal cancer, thecomatosis, androblastoma, endometrium hyperplasy, endometriosis, embryoma, fibrosarcoma, Kaposi's sarcoma, hemangioma, cavernous hemangioma, angioblastoma, retinoblastoma, astrocytoma, neurofibroma, oligodendroglioma, medulloblastoma, ganglioneuroblastoma, rhabdomyosarcoma, hamartoblastoma, osteogenic sarcoma, leiomyosarcoma, thyroid sarcoma, Wilms tumor.

ABSTRACT

The invention provides an anti-TRAIL-R1 and TRAIL-R2 antibody.

Anti-TRAIL-R1 and R2 antibodies or functional fragments thereof, having at least one property selected from the following (a) to (c) of:

(a) having activity to induce apoptosis in carcinoma cells expressing TRAIL-R1 and/or TRAIL-R2;

(b) not having effect on normal human cells (except for hepatic parenchymal cells) expressing TRAIL-R1 and/or TRAIL-R2; and

(c) not inducing human hepatocyte toxicity.

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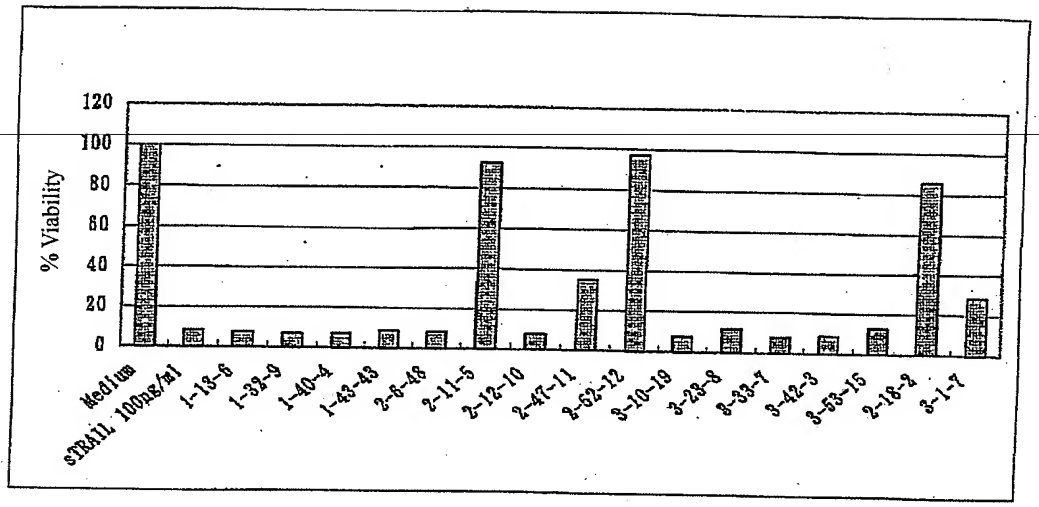
Fig. 1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100
80
60
40
20
0
Medium
sTRAIL 100ng/ml
1-18-6
1-39-9
1-40-4
1-43-43
2-6-48
2-11-5
2-12-10
2-47-11
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Fig. 2

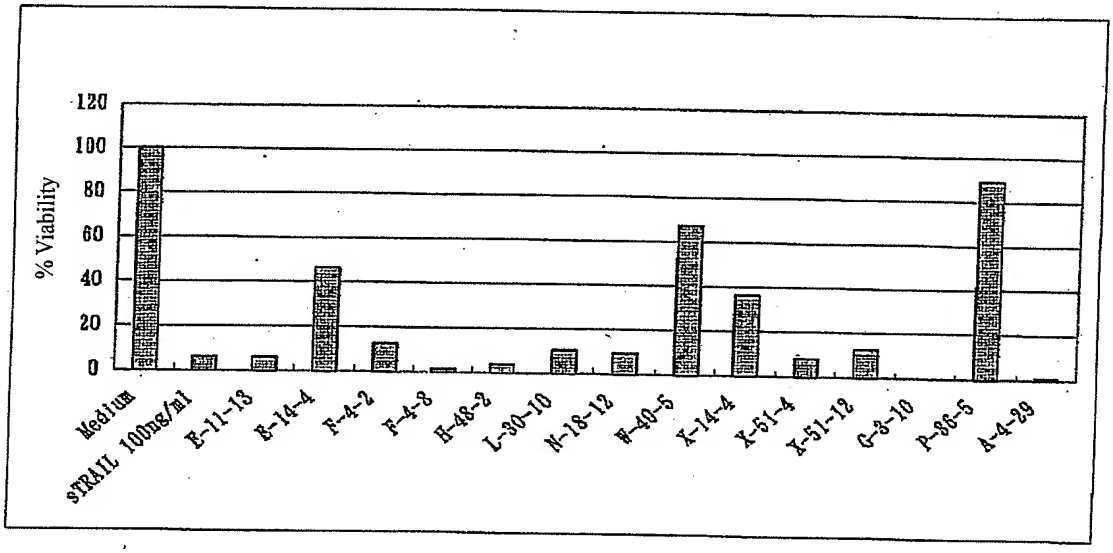
% Viability
120
100
80
60
40
20
0
Medium
sTRAIL 100ng/ml
E-11-13
E-14-4
F-4-2
F-4-8
H-48-2
L-30-10
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Fig. 3

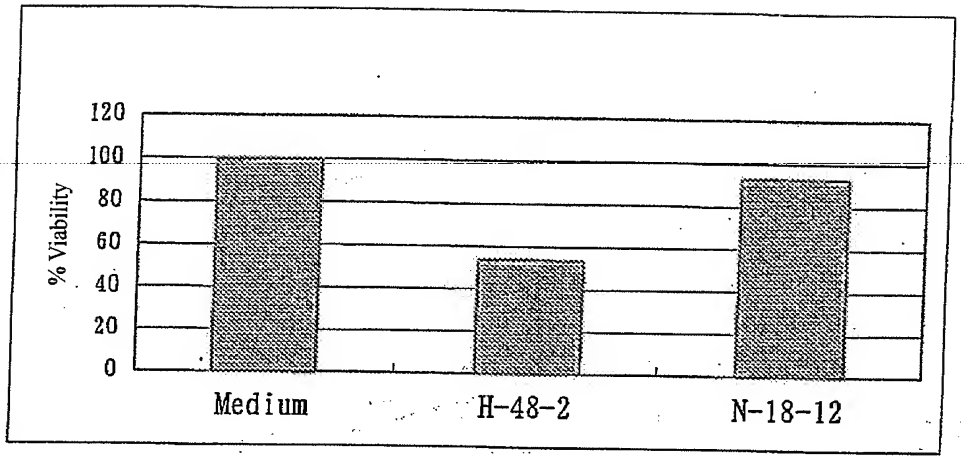
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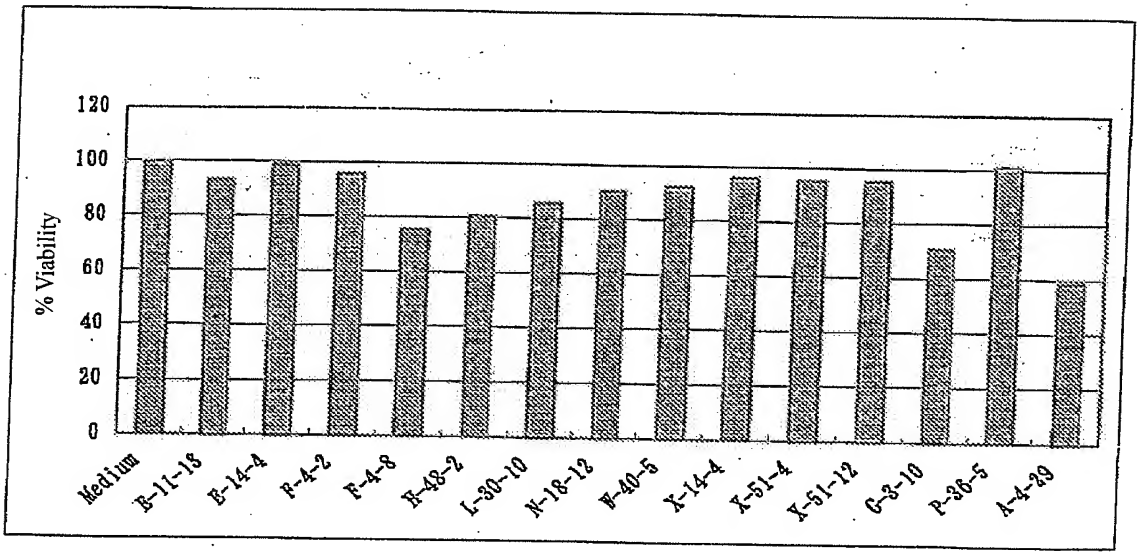
Fig. 4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